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Isolation of candidate genes for apomictic development in buffelgrass (*Pennisetum ciliare*)

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Abstract Asexual reproduction through seeds, or apomixis, is a process that holds much promise for agricultural advances. However, the molecular mechanisms underlying apomixis are currently poorly understood. To identify genes related to female gametophyte development in apomictic ovaries of buffelgrass (*Pennisetum ciliare* (L.) Link), Suppression Subtractive Hybridization of ovary cDNA with leaf cDNA was performed. Through macroarray screening of subtracted cDNAs two genes were identified, *Pca21* and *Pca24*, that showed differential expression between apomictic and sexual ovaries. Sequence analysis showed that both *Pca21* and *Pca24* are novel genes not previously characterized in plants. *Pca21* shows homology to two wheat genes that are also expressed during reproductive development. *Pca24* has similarity to coiled-coil-helix-coiled-coil-helix (CHCH) domain containing proteins from maize and sugarcane. Northern blot analysis revealed that both of these genes are expressed throughout female gametophyte development in apomictic ovaries. In situ hybridizations localized the transcript of these two genes to the developing embryo sacs in the apomictic ovaries. Based on the expression patterns it was concluded that *Pca21* and *Pca24* likely play a role during apomictic development in buffelgrass.

Keywords Apomixis · Apospory · Embryo sac · Gene expression · Megagametogenesis · Ovary

Abbreviations

ASGR	Apomixis-Specific Genomic Region
CHCH	(coiled coil-1) (helix-1) (coiled coil-2) (helix-2)
MMC	megaspore mother cell
SSH	suppression subtractive hybridization

Introduction

Apomixis is a mode of asexual reproduction characterized by the production of clonal seeds through parthenogenetic development of an unreduced egg. Thus the important features of apomictic development are the absence of meiosis (apomeiosis) and fertilization-independent development of the egg cell. Apomixis is broadly divided into three categories: adventitious embryony, diplospory and apospory (Nogler 1984; Ozias-Akins 2006). In adventitious embryony, also known as sporophytic apomixis, a somatic cell from the nucellus or integument differentiates into an embryo. In these species apomictic and sexual development co-exist since successful adventitious embryo development relies on the sexual endosperm. Diplospory and apospory are forms of gametophytic apomixis whereby the apomictic cells go through a gametophytic phase of development to form unreduced embryo sacs. This process mimics sexual reproduction in that a megagametophyte is formed, however meiosis does not occur. In diplospory the

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megaspore mother cell switches from sexual development to apomictic development at varying times during sporogenesis. In apospory, the aposporous initials develop from the nucellar cells generally in the region surrounding the megaspore mother cell and directly initiate gametogenesis to form an unreduced embryo sac. As in sexual development, endosperm is required for the normal growth of apomictic embryos and in many pseudogamous apomictic species fertilization of the central cell is required for the development of endosperm (Koltunow et al. 1995). Fertilization of the central cell is not required in certain apomictic species where the development of endosperm is autonomous.

Apomixis is an important trait for investigation not only from a plant development perspective, but also for crop improvement. Since clonal propagation through apomixis can lead to the fixing of a genotype, it can be an important tool for various plant breeding strategies (Hanna 1995). Most importantly, apomixis can be used for fixation of hybrid vigor in crops where heterosis has been exploited to develop F_1 hybrid varieties (Spillane et al. 2004). However, many hurdles remain in utilizing the potential of apomixis in field crops since it is mostly prevalent in the wild relatives of cultivated species (Savidan et al. 2001). Although genetic studies in many species have identified apomixis as a simply inherited dominant trait (Bicknell et al. 2000; Ozias-Akins et al. 1998; Sherwood et al. 1994), its transfer through hybridization to related crop species has not been successful (Savidan 2001). An understanding of the molecular mechanisms involved in apomictic development may facilitate transfer of this trait into agronomically important crops.

Considerable information is now available on the molecular mechanisms of sexual reproduction in terms of ovule and female gametophyte development (Yadegari and Drews 2004), but not much is known about the molecular processes that distinguish apomixis from sexual development (Bicknell and Koltunow 2004). Many previous studies have compared gene expression during sexual and apomictic development to identify genes associated with apomixis (Pessino et al. 2001; Rodrigues et al. 2003; Vielle-Calzada et al. 1996). However, there is only one previous report of a gene expressed in apomictic, but not sexual, ovules (Chen et al. 1999, 2005). The conclusion from these studies is that sexual and apomictic developments are closely related pathways (Bicknell and Koltunow 2004). Further evidence for this hypothesis was provided by analyzing the expression of several Arabidopsis genes involved in sexual development in apomictic and sexual lines of *Hieracium* (Tucker et al. 2003). The expression of these reporters indicated that apomictic and sexual pathways in *Hieracium* have very similar gene expression profiles and may thus share common regulatory features. However, the fact remains that apomictic development differs from sexual development in two key developmental aspects i.e. apomeiosis and parthenogenesis. Important changes in gene expression are expected to underlie these crucial developmental irregularities even if apomixis can be considered a deregulation of sexual pathway as suggested by several authors (Grimanelli et al. 2003; Koltunow and Grossniklaus 2003).

In the absence of a tractable genetic system in apomictic species, screening of cDNA libraries is a viable approach to identify genes related to apomictic development. In this study the comparison of gene expression between the sexual and apomictic modes of development in buffelgrass is reported. With the aim of isolating genes specific to the female gametophyte of apomictic plants, cDNA libraries were constructed from ovaries following subtraction with leaf cDNA. These libraries were screened with macroarrays and Northern blots. Two genes were identified, *Pca21* and *Pca24*, that are differentially expressed between the sexual and apomictic ovaries. The expression patterns, sequence analysis and the putative roles of these genes in apomictic development are discussed.

Materials and methods

Plant materials

Full-sib obligate sexual and obligate apomictic lines from a complex buffelgrass cross (Jessup et al. 2002) were grown in a USDA-ARS greenhouse at College Station for use in this study. Pistils with ovaries corresponding to sporophytic and gametophytic stages of ovule development were dissected from apomictic plants. The stigma and styles were removed from each pistil leaving only the ovary. The ovaries were categorized into four different developmental stages as described by Rodrigues et al. (2003). Stage determination was based on cytological analysis of pollen mother cells in the anthers and morphological analysis of ovaries as described by Vielle-Calzada et al. (1996). Stage I ovaries comprise the pre-meiotic stage with a differentiating megaspore mother cell (MMC). Stage II corresponds to ovaries with the megaspore mother cell undergoing meiosis (in the sexual line). Stage III and IV ovaries represent gametogenesis and mature female gametophyte respectively. Ovaries from stages I and II were pooled to encompass the period of megasporogenesis that also corresponds to the development of aposporous initials. Young leaves from sexual or apomictic plants were used as the source for leaf RNA.

RNA extraction and cDNA synthesis

Dissected ovaries were collected in 25–30 μl of RNAqueous lysis buffer (Ambion) and stored at –80°C until used for RNA extraction. Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Following extraction the RNA sample was dissolved in 15–20 μl diethyl pyrocarbonate (DEPC)-treated water. cDNA synthesis was performed with the SMART cDNA synthesis kit (BD Biosciences) with 600 ng of total RNA following the manufacturer's instructions.

Suppression subtractive hybridization (SSH)

The SSH procedure was performed with the PCR Select cDNA Subtraction kit (BD Biosciences) as directed by the manufacturer, except a one and half fold excess of driver cDNA was added to the first and second hybridizations. Sporogenesis and gametogenesis stage apomictic ovary cDNA was used as tester and cDNA synthesized from leaf total RNA was used as driver. Following hybridization and PCR, products were fractioned on a 1% agarose gel and cloned into pCR2.1 TOPO (Invitrogen) cloning vector.

Screening of SSH-library cDNA macroarrays

The cloned cDNA inserts were amplified and spotted onto nylon membranes in quadruplicate to produce macroarrays. To amplify cDNA inserts, individual colonies were first cultured overnight in 200 μl LB in 96-well plates. PCR was performed with M13 forward and reverse primers using 1 μl of overnight bacterial culture as template. The inserts were denatured with 0.2 M NaOH for 5 min at 95°C, and then spotted onto Hybond-N filters (AP-Biotech) with a 96-pin print head tool. The DNA was fixed using a UV cross-linker and the filters were then stored at room temperature until used for hybridizations. The blots were probed with ^{32}P-dCTP-labeled cDNA transcribed from total RNA of ovaries and leaves using the Superscript II Reverse Transcription System (Invitrogen) in a reaction using 10 μg of total RNA primed with oligo dT. Hybridizations were carried out at 60°C overnight in hybridization buffer that contained 10% dextran sulphate, 1% SDS, 1 M NaCl and 100 μg/ml of denatured salmon sperm DNA. Following hybridization, the blots were washed with 0.1X SSC and 0.5% SDS at 60°C.

Northern analysis

Five to ten micrograms of total RNA were separated on a denaturing glyoxal agarose gel and blotted to HyBond-N filter (A-P Biotech). Probes were prepared from cDNAs using a DNA random-labeling kit (RadPrime, Invitrogen). Hybridizations and washings were as described above, except at 65°C.

Quantitative reverse-transcription real-time PCR (QPCR)

Four micrograms of total RNA for each sample (leaf and ovary) was digested with 3.5 u of DNAse I for 30 min, then re-extracted with TRIzol (Invitrogen). cDNA was synthesized with the Superscript III kit using random hexamers according to the manufacturer's instructions (Invitrogen). The cDNA was diluted 1:5 and 10 µL real-time PCR reactions were run in duplicate on an ABI 7900HT, with corresponding -RT controls, using the SYBR Green Jumpstart kit (Sigma). Target cT values were determined and normalized to 18s rRNA cT values. Dissociation curves were analyzed for each reaction to ensure that there were no non-specific amplification products or primer-dimers.

In situ hybridizations

Spikelets with ovaries at the gametogenesis stage were fixed overnight in 4% formaldehyde and 0.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.0) at 4°C. Fixed tissues were dehydrated and embedded in TissuePrep (Fisher Scientific) and sectioned at a thickness of 10 μm with a rotary microtome. The sections were placed on poly-D-lysine coated slides overnight at 45°C. Digoxigenin-labeled sense and antisense probes were transcribed from 100 to 150 bp cDNA fragments cloned in pBluescript KS+ plasmid (Stratagene) using digoxigenin-11-UTP (Roche) and T3 or T7 Ribozyme in vitro transcription kit (Promega). For hybridization, the sectioned samples were de-paraffinated in xylene and rehydrated in a graded ethanol series. Samples were then treated for 30 min at 37°C with proteinase K (100 μg/μl). Prehybridization, hybridization, washes and detection were carried out using the Roche Digoxigenin Nucleic Acid Detection kit according to the methods described in the manufacturer's technical manual.

Plant DNA isolation and southern analysis

Total genomic DNA was isolated from leaf tissue following the method of Dellaporta et al. (1983). Ten micrograms of DNA was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel and blotted onto a Hybond-N filter membrane. Probes were prepared from PCR amplified cDNA fragments through random labeling as described for RNA blots. Hybridization and washing conditions were similar to those described for macroarray blots.

Results

Identification of genes expressed during female gametophyte development

To identify genes differentially expressed during sexual and apomictic development in buffelgrass, leaf-subtracted cDNA libraries were constructed from apomictic ovaries at the sporogenesis and gametogenesis stages. In total, 1152 clones were obtained following cDNA subtraction and ligation of amplified products. Macroarrays were employed to screen the subtracted libraries to identify genes preferentially expressed in the ovaries. Figure 1 shows representative macroarray blots of cDNA from leaf-subtracted gametogenesis stage apomictic ovaries probed with apomictic ovary, sexual ovary and leaf cDNA. It was observed that the pattern of gene expression was very similar not only in both the apomictic and sexual ovaries but also in leaves. Detailed studies on the successful application of SSH show that for effective enrichment of differentially expressed genes, the concentration ratio of these differentially expressed genes needs to be more than 5 (Ji et al. 2002). In the current study, the expression differences between some of the common genes in leaves and ovaries are likely not different enough for efficient subtraction even though these genes appear to be expressed at higher levels in leaves as shown by macroarrays (Fig. 1). However, based on the screening with macroarrays a number of cDNAs were identified that are differentially expressed between the ovary and leaf both at the sporogenesis and gametogenesis stages. For instance, from Fig. 1 it can be observed that leaf and ovary probes hybridized to many of the same spots, though the relative intensity often varied. Conversely, some cDNAs were specific to ovaries as indicated by the boxed areas in Fig. 1. Each box encloses a 4 replicate spot group of an independent cDNA.

In total 121 cDNAs were selected from the SSH libraries based on initial macroarray screening and their expression was subsequently analyzed by Northern blot and QPCR. Expression in leaves and/or ovaries was detected with 72 of the 121 cDNAs (Table 1). No expression was detected with the remaining 49 cDNAs, which were largely identified from spots that showed very low signal on the microarrays. Of the 72 genes with apparent expression, 57 showed differential expression between ovary and leaf (higher or exclusive expression in ovary), giving a total positive rate of 79%. Sixteen cDNAs were additionally picked at random from the SSH libraries and analyzed by QPCR. From this random sample only three genes showed differential expression in the ovaries compared to leaves, giving a positive rate of 23%. Therefore, initial macroarray screening substantially increased the efficiency of identifying genes differentially expressed between ovaries and leaves. Examples of cDNAs expressed in ovaries but not in leaves are; cDNA *1H4*, which was obtained from sporogenesis stage ovaries, and cDNA *1H3*, which was identified from the mature stage ovary cDNA library (Fig. 2). The level of expression of these two clones was similar in apomictic and sexual ovaries. Based on the macroarray and Northern screening, a total of three cDNAs were identified that exhibited differential expression between the apomictic and sexual ovaries. cDNA *4B10*, isolated from the sporogenesis stage library, was exat higher levels in apomictic vs. sexual ovaries (Fig. 2). Two other genes, *Pca21* and *Pca24*, showed striking differences in expression between apomictic and sexual ovaries and their detailed analyses are presented below.

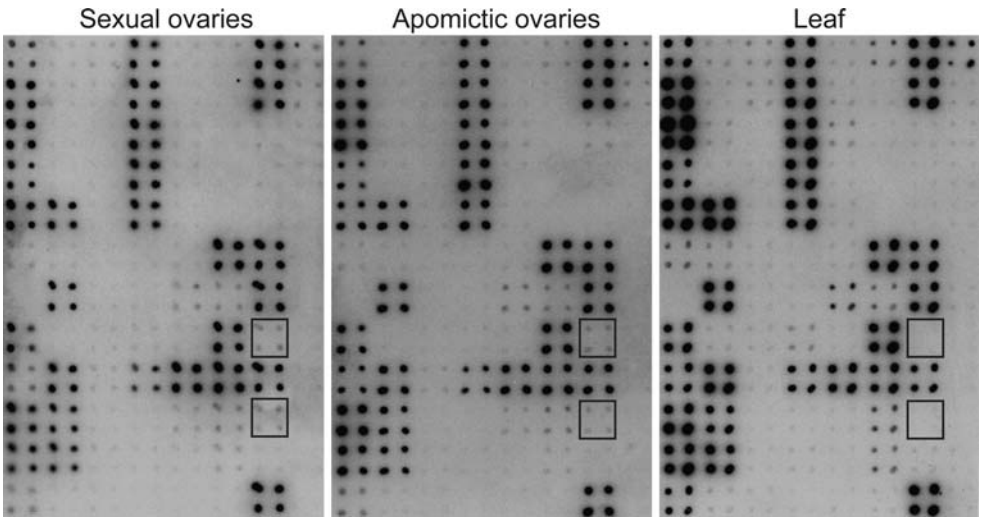

Fig. 1 Example macroarray blots probed with sexual ovary, apomictic ovary and leaf cDNA. Boxed spot groups are differentially expressed between ovaries and leaves

Table 1 Efficiency of macroarray screening of, and random clone selection from, SSH libraries in identifying genes differentially expressed in ovaries versus leaves

	Randomly picked cDNAs Sporophytic library—QPCR	cDNAs identified by initial macroarray screening			
		Sporophytic library—QPCR	Sporophytic library—Northern blot	Gametophytic library—Northern blot	Total
Total cDNAs analyzed	16	10	69	42	121
cDNAs with detectable expression	13	9	49	14	72
Differentially expressed in ovary versus leaf	3	8	39	10	57
% cDNAs analyzed with differential expression	23.1	80	79.6	71.4	79.2

Pca21 and *Pca24* are differentially expressed between apomictic and sexual ovaries

Based on the macroarray screening of subtracted cDNA from apomictic ovaries at the sporogenesis stage two cDNA clones *Pca21* and *Pca24* were identified that differed in expression between the apomictic and sexual ovaries. The expression of these two cDNA clones was analyzed in detail with respect to their timing and tissue-specificity in the apomictic and sexual genotypes. The expression of *Pca24* was specific to apomictic ovaries and no expression was detected in the sexual ovaries (Figs. 3 and 4). *Pca24* transcript also was not detected in the spikelet tissue of apomictic plants from which the pistils had been removed (not shown). *Pca21* was predominantly expressed in the apomictic ovaries but a low level of expression could be detected in the sexual ovaries (Figs. 3 and 4). The *Pca21* cDNA was also specific to ovaries and its transcript was not detected in leaves or other floral organs (Figs. 3 and 4). Analyses of the transcripts at pre-meiotic, meiotic and mature inflorescence stages showed that both these genes were expressed throughout sporogenesis and gametogenesis (Fig. 4).

It is likely that genes with apomixis-related functions should be expressed in the aposporous (nucellar) initials and the embryo sacs derived from these cells. Since RNA blots showed that *Pca21* and *Pca24* are expressed throughout female gametophyte development, in situ hybridizations were carried out on ovaries at the gametogenesis stage to localize their expression. In situ hybridization with antisense *Pca21* and *Pca24* probes revealed that both genes are expressed in dividing nuclei in embryo sacs of the apomictic ovaries (Fig. 5). No signal was detected in hybridizations with the sense probes.

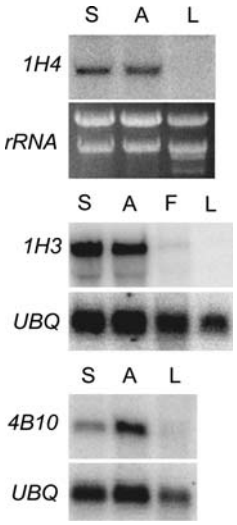

Fig. 2 Examples of Northern blots of apomictic (A) and sexual (S) ovary, floret without ovary (F) and leaf (L) RNA confirming expression patterns of cDNAs identified from macroarrays. *1H4*, *1H3* and *4B10* are independent cDNA fragments used as probes. Some blots were re-probed with *Ubiquitin* (*UBQ*) to verify equal loading

Sequence and bioinformatics analysis of *Pca21* and *Pca24*

Based on the RNA blot analysis the transcript size of both *Pca21* and *Pca24* is estimated to be about 900 bp. The total

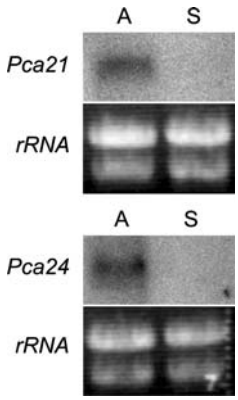

Fig. 3 Northern blots of apomictic (A) and sexual (S) ovary RNA probed with *Pca21* and *Pca24*

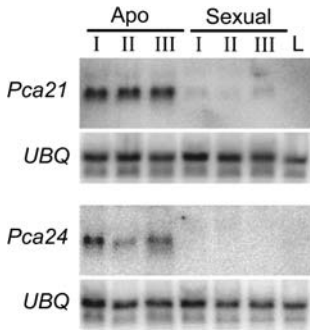

Fig. 4 Northern blots of apomictic (A) and sexual (S) spike/inflorescence RNA at different stages of development probed with *Pca21* and *Pca24*. Blots include leaf RNA (L) as a control. Stages are; sporogenesis (I), gametogenesis (II) and mature ovaries (III)

sequence obtained for *Pca21* cDNA from the initial cDNA and 5′ and 3′ RACE is 830 bp. The *Pca21* sequence has been deposited in GenBank under accession number EF517497. This sequence contains an ORF of 134 amino acids that is similar in size to a wheat cDNA clone (BJ267504) with which it shares high homology. The total sequence obtained for *Pca24* is 621 bp that represents a partial cDNA and encodes a partial ORF of 149 amino acids. The *Pca24* sequence has been deposited in GenBank under accession number EF517498. Blast analysis of *Pca21* and *Pca24* showed that these are novel genes with no significant similarity to previously characterized genes. *Pca21* shows high homology to two wheat cDNAs (accession numbers BJ267504 and BG262694) as indicated by a protein sequence alignment (Fig. 6A). A rice genomic sequence on chromosome 2 also possessed homology to a small fragment of *Pca21*, but further analysis indicated this sequence was likely a retrotransposon chimera. At the amino acid level the Pca21 protein does not show significant similarity to any protein in the NCBI database and a domain search did not identify any domains in Pca21 or the translated wheat cDNAs. The Pca24 sequence (Fig. 6B) shows homology to (coiled coil-1) (helix-1) (coiled coil-2) (helix-2) (CHCH) domain containing maize and sugarcane cDNAs. The partial amino acid sequence of the *Pca24* cDNA also does not show significant similarity to any protein in the NCBI database. A comparison with the Pfam database using MotifScan (Falquet et al. 2002) identified a CHCH domain near the 3′ end. While the CHCH domain homology is not strong (E-value = 0.0093), Pca24 does contain the four-conserved cysteine residues characteristic of the CHCH domain, two of which are located in each helix. The two cysteine residues in each helix are separated by nine amino acids (C–X_9–C) as is the case with many

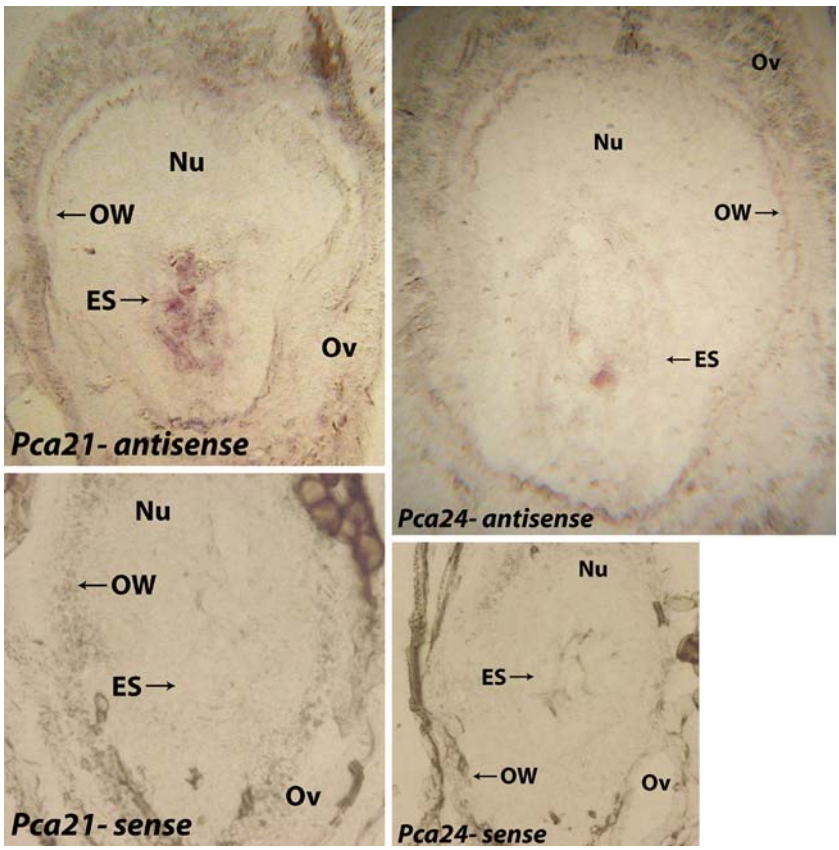

Fig. 5 In situ hybridizations of apomictic ovaries at gametogenesis stage (dividing or developing embryo sacs) probed with *Pca21* and *Pca24*

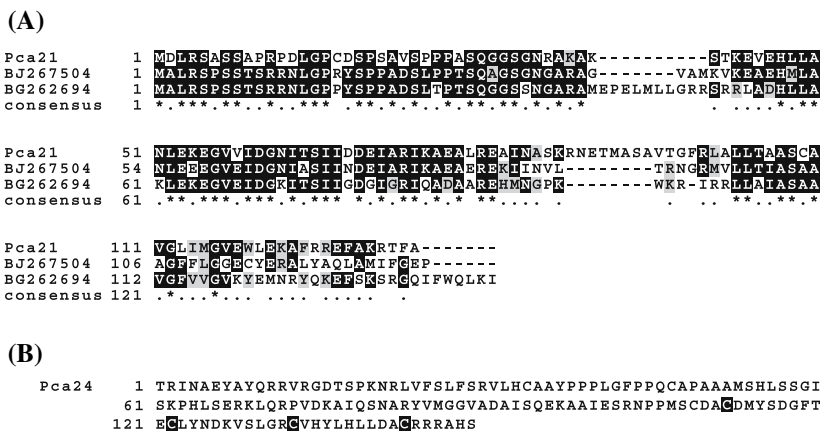

Fig. 6 ClustalW alignment of predicted Pca21 and homologous wheat protein sequences (**A**). Hypothetical partial amino acid sequence of *Pca24* showing four conserved cysteine residues characteristic of CHCH domains (**B**)

CHCH domain containing proteins (Westerman et al. 2004).

Southern blot analysis

Southern blot analysis was performed to study the genomic organization of *Pca21* and *Pca24* in buffelgrass and *Pennisetum squamulatum* and *Pennisetum glaucum*, closely related apomictic and sexual species respectively. The apospory-linked locus in buffelgrass has been reported to be hemizygous (Jessup et al. 2003). Therefore it is likely that the genes involved in apospory are linked to this locus and hence present only in apomictic genotypes. A DNA blot probed with *Pca21* showed that there is only one copy of this gene in the apomictic buffelgrass whereas two or more copies may be present in the sexual genotype (Fig. 7). However, the opposite appears to be true for *P. squamulatum* and *P. glaucum* that have two copies and one copy respectively. Although the polymorphism is expected between the two *Pennisetum* species, polymorphism was also detected between the apomictic and sexual genotypes

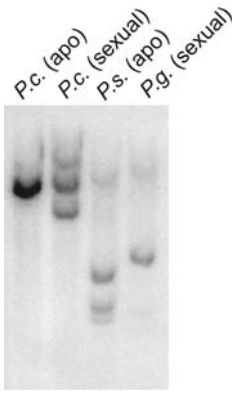

Fig. 7 Southern blot of apomictic and sexual *Pennisetum ciliare*, apomictic *Pennisetum squamulatum* and sexual *Pennisetum glaucum* genomic DNA digested with *Eco*RI and probed with *Pca21*

of buffelgrass. Preliminary mapping of *Pca21* and *Pca24* showed that neither gene is linked to the Apomixis-Specific Genomic Region (ASGR) in buffelgrass. A PCR fragment of *Pca24* was amplified from the apomictic parent but not the sexual parent in mapping efforts.

Discussion

Molecular control of apomictic development has intrigued researchers for many years and the investigation of gene expression during apomictic development has been the focus of many previous studies. Until now, the only report of a gene specifically expressed during apomictic development is *ASG-1* in *Panicum* (Chen et al. 1999; Chen et al. 2005). Other studies have not been conclusive on the expression of genes that were initially identified on the basis of differential expression (Guerin et al. 2000; Pessino et al. 2001; Vielle-Calzada et al. 1996). Macroarray analysis of 1152 cDNAs from apomictic buffelgrass lines in this study showed that gene expression is conserved to a great extent not only between sexual and apomictic ovaries but also between ovaries and leaves. These observations lend support to the view that apomictic development may be largely the result of altered expression of genes involved in sexual development. Carman (1997) has suggested that asynchronous expression following polyploidization of genes involved in female gametophyte development may cause apomixis. Recent investigations of gene expression patterns during apomictic development have used this theory as a basis for experimental design and identified genes that are common to sexual and apomictic pathways but that differ in spatial or temporal expression. *PpSERK* a candidate apomictic gene in *Poa pratensis* is expressed both in sexual and apomictic genotypes but exhibits dissimilar spatial expression in the developing ovules of the two pathways (Albertini et al. 2005). In sexual genotypes the expression of this gene was detected in the MMC,

whereas in the apomicts its transcript was localized in the nucellar cells neighboring the MMC. Thus, it may be inferred that *PpSERK* is involved in embryo sac development in both sexual and apomictic pathways. However, apospory involves deviation from two fundamental events of sexual development that are likely to require expression of some unique genes. Since the aposporous cells also undergo gametogenesis they are expected to express the genes required for gametogenesis during sexual development. Spillane et al. (2001) suggested that expression of a combination of genes both common and specific to sexual and apomictic pathways results in apomixis. According to the hypothesis of Matzk et al. (2005), once the aposporous initials are selected, the cascade for gametogenesis as in sexual development begins and the two pathways converge, expressing similar genes during the process.

Pca21 and *Pca24* are novel genes

Pca21 and *Pca24* were identified from a sporogenesis-stage subtracted cDNA library as transcripts preferentially (*Pca21*) or exclusively (*Pca24*) expressed in apomictic ovaries. Sequence analysis showed that both are novel genes that have not been previously characterized in plants. *Pca21* shows high homology to two wheat genes (locus Ta.4513 and Ta.10950) and rice genomic sequence of chromosome 2. The two wheat genes are very similar and may be copies of the same gene. Both the wheat genes are similar in size to *Pca21* and encode hypothetical proteins of 130–142 aa which is similar to the *Pca21* ORF. A protein domain search of Pca21 and the translated wheat genes did not find any putative domains. However, based on the weak homology of *Pca21* and the wheat cDNAs to sequences in the database, these genes may contain an ATP-binding domain.

Pca24 shows strong homology to maize and sugarcane cDNAs that encode predicted proteins with CHCH domains. The CHCH domain is present in many diverse eukaryotic proteins including those involved in mitochondrial translation, estrogen and ethanol-induced proteins and NADH: ubiquinone oxireductase-like proteins (Westerman et al. 2004). Some of these proteins have been shown to exhibit a developmental pattern of expression and have important developmental roles (Yang et al. 2000). The functional significance of the CHCH domain is unknown, but its presence in a large number of diverse proteins suggests a conserved and potentially important role. Although a search identified only a weak match to a CHCH domain in the *Pca24* protein, the four cysteine residues are present as a C–X_9–C motif separated by 10 amino acids as is the case with most CHCH domain containing proteins (Westerman et al. 2004). The absence of a canonical CHCH domain suggests that the *Pca24* protein is different from the CHCH domain-containing proteins and may have a different function. While the function of the CHCH domain is not understood, it has been suggested that the four cysteines may act as a ligand of a metal-binding domain (Westerman et al. 2004). Since the four cysteine residues are intact in the predicted Pca24 protein, it may have a metal binding property similar to that of zinc-finger and related proteins that bind metals via cysteine residues.

Pca21 and *Pca24* are expressed in a pattern expected for genes involved in apomixis

Northern analysis and in situ hybridizations showed that *Pca21* and *Pca24* have very similar expression patterns. Both genes are expressed throughout female gametophyte development in apomictic ovaries. Although *Pca21* is predominantly expressed in the apomictic ovaries, a low level of expression was detected in the sexual ovaries suggesting that this gene may also have a role in sexual development. The expression profiles of two wheat genes that show high homology to *Pca21* also suggest a role during sexual development. Information about the expression of these genes can be obtained from the Unigene EST profile viewer (Pontius et al. 2003). The wheat cDNA BJ267504 (locus Ta.4513) was isolated from a pistil cDNA library at heading stage, which corresponds to sporogenesis or gametogenesis stages. The EST profile data showed flower-specific expression of this gene. The second wheat cDNA that shows high homology to *Pca21*, BG262694 (locus Ta.10950), was isolated from a 5–10 DAP spike cDNA library and its EST expression profile was specific to flowers and inflorescence. Thus, the expression patterns of *Pca21* and homologous wheat cDNAs suggests that they may be required for female gametophyte development in both sexual and apomictic genotypes. The difference in the level of expression between the sexual and apomictic ovaries might be attributed in part to the presence of multiple embryos sacs in the later. Southern analysis also showed that the *Pca21* gene is present in both the apomictic and sexual genotypes of buffelgrass as well as in *P. squamulatum* and *P. glaucum*.

Pca24 transcript was detected only in embryo sacs of apomictic ovaries supporting an apomixis-specific function. Its similarity to CHCH domain-containing proteins also indicates that this gene may have a developmental role. Although no CHCH domain proteins have been analyzed in plants, a search of the Arabidopsis and rice databases shows several genes encoding for proteins that contain a CHCH domain. It is likely that *Pennisetum* also has a number of CHCH domain proteins, and *Pca24* may have evolved from these genes and acquired an apomixis-related function. In this context, *Pca24* may be an apomixis-specific allele of a CHCH domain-like protein in

buffelgrass. This gene may have acquired an apomixis-specific role either through a change in its expression pattern or through structural divergence from the progenitor gene, or both. Since Southern data on the genomic organization of *Pca24* in apomictic and sexual genotypes is inconclusive, it is not possible to confirm if a sexual allele exists for this gene. However, PCR and expression results clearly demonstrate a divergence in the genome between sexual and apomicts at this locus. Few apomictic development-related genes have been isolated, making it difficult to speculate on the nature of origin of these genes. *ASG-1*, another apomixis-specific gene, is believed to be an allele of a sexual gene since a copy is also present in sexual genotypes (Chen et al. 1999). *PpSERK*, another apomixis-related gene, is present in multiple copies in sexual and apomictic genotypes and is likely to have apomictic and sexual alleles (Albertini et al. 2005). Apomixis-related genes in the ASGR may have evolved from their sexual counterparts outside the ASGR through genome re-arrangements and isolation from genetic recombination (Roche et al. 2001). While *Pca24* does not appear to map to the ASGR, similar mechanisms might have contributed to its evolution.

Role of *Pca21* and *Pca24* in apomixis

Based on the pattern of expression of *Pca21* and *Pca24* genes some speculation can be made regarding their functions in apomictic development. In sexual reproduction a nucellar cell differentiates in to a MMC, which undergoes sporogenesis and gametogenesis to form an embryo sac while the remaining nucellar cells eventually undergo programmed cell death and are consumed for the nutrition of the developing embryo sac (Bhojwani and Bhatnagar 1992; Wu and Cheung 2000). During sexual development the maize protein Multiple archesporial cells1 (Mac1) and the rice protein Multiple sporocytes1 (Msp1) restrict the number of nucellar cells that differentiate into the MMCs that initiate sporogenesis (Nonomura et al. 2003; Sheridan et al. 1996). The mechanisms by which Mac1 and Msp1 limit cells entering the sporogenesis program are not known. However, from the mutant analyses it is evident that these proteins impose a repressive control over the nucellar cells to restrict the number of sporogenic initials to one. These proteins may employ a mechanism similar to that of RegA during germ-soma differentiation in *Volvox carteri* (Schmitt 2003). RegA causes degeneration of somatic cells by repressing the transcription of key genes required for their survival. Consequently, only the germinal cells continue to divide and differentiate (Kirk et al. 1999, Meissner et al. 1999). Since only a few cells of the nucellus initiate apospory, a mechanism similar to that in the sexual pathway likely exists to restrict the number of cells that participate. *Pca21* may thus be the target of a RegA-like regulatory protein and required for further cell division and differentiation, whether it is the MMC or an aposporous initial. The *Pca21* protein in fact shows weak homology to a chloroplast ATP synthase that is the target of RegA (Meissner et al. 1999).

Pca24 on the other hand is an apomixis-specific gene that may have a regulatory role in apomictic development. Its expression was observed throughout apomictic development from aposporous initiation to embryo sac maturation suggesting that this gene may have a role in different stages of apomixis. Alternatively, the early expression of this gene in the aposporous initials may be required for it to function later during embryo sac development as was suggested for *AGP18*, an Arabidopsis gene required for gametogenesis initiation (Acosta-Garcia and Vielle-Calzada 2004). A detailed analysis of the expression of this gene at various stages will shed more light on its role in apomictic development.

Irrespective of their functions, the identification of the apomixis-specific genes *Pca24* and *ASG-1* (Chen et al. 1999, 2005) suggests that a set of unique genes are expressed during apomictic development. Some members of this set of apomixis-specific genes may be part of the complex Apomixis-Specific Genomic Region. However, the set may also include a subset of genes not linked to the ASGR that are regulated by an apomixis master gene or the proposed *Apospory initiator* gene (Matzk et al. 2005). Preliminary mapping data indicates that the *Pca24* gene is not linked to the ASGR in buffelgrass. *Pca24* may thus belong to the subset of apomixis-specific genes that are activated by the apomixis master gene and subsequently play a downstream role in the initiation of apospory or in the integration of various components of the sexual pathway, such as gametogenesis and embryogenesis, into apomictic development. Further detailed functional analysis of *Pca24* will help to define its role in apomictic development and thus offer additional insights into the molecular events associated with apomixis.

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